AD NUMBER

AD840015

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SMUFD D/A ltr, 15 Feb 1972

TRANSLATION NO. 2153

DATE: 10 April 1968

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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

THE MULTIPLICATION AND VIRULENCE OF SEPTICEMIC PATHOGENS WHEN CULTIVATED IN DEFIBRINATED BLOOD

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Izvestiya na Mikrobiologicheskiya Institut na B"lgarskata Akademiya na Naukite (News of the Microbiological Institute of the Bulgarian Academy of Sciences), Vol. 17, Sofia, 1965, pages 155-164

After we had investigated the multiplication and virulence of a number of septicemic pathogens in the organism of infected animals and their carcasses and after it had been established that in septic carcasses there takes place an extraordinarily intense multiplication of these pathogens, accompanied by a stepping up of their virulence to exceptionally high levels -- on the basis of which finding was revealed the true role played by carcasses and materials from dead bodies from the viewpoint of epizootiology, epidemiology and the natural foci of this kind of infection, we set ourselves the problem of investigating how multiplication and virulence proceed in vitro in the blood of various species of animals. Investigations along this line were undertaken with a view to preparing for the next stage of the work from the point of view outlined above, viz. to ascertain whether septicemic and blood pathogens can multiply and increase in virulence in the extraordinarily small amounts of blood which are taken by bloodsucking arthropods in transmissive bacterial infections.

The basis for initiating these investigations was given us by the observations, reported by Merchant and Packer, regarding the increase in virulence of Pasteurellae after cultivation in the blood [1] and the fact well known in epidemiology and epizootiology that Pasteurella pestis multiplies at an extraordinary rate in the blood found in the

esophagi of the fleas which spread this zoonosis.

Experimental solution of the problems thus formulated, viz. investigation of multiplication and change of virulence in blood in vitro and thereafter in the blood taken by blood-sucking arthropods, would no doubt contribute to an even fuller and more scientific clarification of the role and significance of these arthropods in the epizootiology, epidemiology and natural foci of a number of infectious diseases.

Material and Methods

The technique used is to a great extent similar to that in the other works on the multiplication and virulence of septicemic pathogens in infected animals and their carcasses, but adapted to the case. Inoculation is from 24-hour bouillon cultures into defibrinated sheep and swine blood. After cultivation at 37° for 24 hours the culture is sowed on ordinary bouillon, which is used as a 24-hour culture [illegible] the investigated bacterial species on [illegible] ing (initial) virulence of the investigated mined beforehand, we looked for the degree virulence during a 24-hour period as well as [illegible] dynamics (at 24, 48, 72 hours and so on). At the same hours a count was made of the multiplication of microorganisms either by counting the colonies thereof on agar or by direct count in smears of identical thickness (a platinum loop of inoculated blood applied to an area defined by a template).

Strains of Erysipelothrix rhusiopathiae, Listeria monocytogenes, Pasteurella avicida, Pasteurella pseudotuberculosis, Bacterium pyocyaneum and Salmonella typhimurium underwent analysis from this point of view.

We set forth the obtained results in sequence.

Results

Erysipelothrix rhusiopathiae. During 24-hour culturing at 37° in defibrinated sheep blood the erysipelas bacterium attained almost the same degree of virulence as in the carcasses of experimentally inoculated animals [4]. With a starting virulence of $2 \cdot 10^{-3}$ virulence increased in 24 hours to $1.2 \cdot 10^{-5}$ with this difference that whereas the strains isolated from carcasses induce death in white mice on the 4th or 5th day, in the case of strains isolated from blood death ensued most frequently on the 8th day. The reasons for this delay in the time of death are the subject of further study.

The virulence of erysipelas bacteria isolated from carcasses of experimentally inoculated animals and birds, as established in the preceding studies, is at its highest by the 5th-9th hour after death (in the case of carcasses kept at a temperature of 18-22°). Later it begins to decline due to the suppression of certain vital functions of the bacterial cells, which sets in and proceeds as a result of intensified putrefactive processes. In view of this, we were interested in investigating how virulence would change in a medium lacking septic microorganisms, such as blood taken under sterile conditions. For this purpose we undertook to investigate the dynamics of this process during protracted culturing in blood, as well as the extent to which any changes in virulence would coincide with the observed increase in virulence in a septic erysipelas carcass. It was established that with 24-hour culturing in defibrinated sheep blood taken under sterile conditions the virulence of Ery. rhusiopathiae increases to $1.2 \cdot 10^{-5}$ (a 60-fold increase in comparison with starting virulence); with 48-hour culturing to $2.5 \cdot 10^{-5}$ (a 120-fold increase); with 72-hour culturing to $5.1 \cdot 10^{-5}$ (a 255-fold increase); with 120-hour culturing to 10^{-6} (a 500-fold increase); with 144-hour culturing $2 \cdot 10^{-6}$ (a 1000-fold increase). Thereafter it gradually declined, at the 192nd hour reaching the level at 120 hours (10^{-6}) and continuing to diminish. More protracted culturing in blood leads to the production of R forms. Clearly, the possibilities of an increase in the virulence of erysipelas bacteria are much greater in blood in vitro than in a septic carcass.

Simultaneously with the dynamics of the change in virulence, the dynamics of the multiplication of Ery. rhusiopathiae in defibrinated sheep blood was determined. At the above-indicated times cultures of 1:1,000,000 dilutions of bacteria cultivated in blood were made on agar in Petri dishes. From the number of colonies we determined the number of bacteria per ml. As a result of the thus-conducted analysis the number of erysipelas bacteria per ml of blood went as high as 2.5 billion in 24 hours, 1.15 billion in 48 hours, 570 million in 72 hours, 400 million in 96 hours, 250 million in 120 hours, and 400 million in 168 hours after which it gradually dropped and went to 200 million at the 240th hour. It can be seen that the number of erysipelas bacteria is greatest at the 24th hour, after which it declines while virulence increases to the 144th hour, that is, the intensification of virulence by blood is not directly proportional to multiplication.

The results in the case of culturing in swine blood are identical with those above.

Listeria monocytogenes. As in the case of Erysipelothrix rhusiopathiae, here likewise we undertook to follow through the changes in virulence during culturing in defibrinated sheep and swine blood. During a 24-hour stay in the thermostat the Listeriae in swine and sheep blood increased their virulence to a degree close to the maximum in a putrescent septic carcass. Given a starting virulence of $3 \cdot 10^{-2}$, it reached 10^{-5} in 24 hours, with mice here again dying with a certain lag of from 1 to 3 days. With more protracted culturing in blood virulence increases significantly, viz. 3000 fold in 24 hours, 10^{-6} in 48 hours (a 30,000-fold increase) with a gradual increase at the 72nd and the 96th hour, reaching 10^{-7} (a 300,000-fold increase) at the 120th hour, and 10^{-8} (a 3,000,000-fold increase) at the 144th hour, with a gradual decline during later hours. As in the case of Ery. rhusiopathiae, virulence in the case of Listeriae rises smoothly and is maximal at the 144th hour with a 1000-fold increase in comparison with the 24th hour, i. e. the change in virulence takes a course of the type characteristic of the erysipelas pathogen. The same thing holds for multiplication in blood -- the maximum is reached by the 24th hour with a gradual decline in the number of Listeriae per ml of blood during subsequent hours.

Pasteurella avicida. In determining the change in virulence of the chicken-cholera pathogen in carcasses of experimentally inoculated mice, rabbits, hens and pigeons, we established an increase in virulence to an exceptionally high degree [5]. It was of interest whether this paradoxical increase in virulence would be duplicated with culturing in blood.

For convenience we worked with the vaccine strains of Tonev and Zhekov.

In the event of 24-hour cultivation in defibrinated sheep blood taken under sterile conditions, the virulence of Pasteurellae increases to an extent almost identical with the change of virulence in putrid septic carcasses. With initial virulence of 10^{-1}, the "Tonev" strain increased its virulence in 24 hours to 10^{-16}, and the "Zhekov" strain from 10^{-2} to 10^{-12}. The mice inoculated with these Pasteurellae died several days later than the mice inoculated with Pasteurellae isolated from carcasses.

In the event of culturing in defibrinated swine blood, virulence reached 10^{-14} in 24 hours with the "Tonev" strain and

10^{-12} with the "Zhekov" strain.

Multiplication of Pasteurellae in blood was at its maximum at the 30th hour (30-40 billion cells per ml of blood) with a gradual decline during subsequent hours (20-25 billion at the 36th-48th hour and 10-15 billion at the 60th-100th hour). And here, just as in the case of erysipelas bacteria and Listeriae, the greatest degree of virulence did not tally in time with the moment of most intense multiplication. Whereas in carcasses the virulence of Past. avicida is maximal at the 12th-24th hour (depending on the temperature at which the carcass is kept) and at the 24th hour in blood, we have the most Pasteurellae per ml of blood at the 30th hour.

It was of interest to know whether Pasteurellae like erysipelas bacteria and Listeriae would increase in virulence if cultured more protractedly in blood. We staged this experiment with a new strain of Past. avicida with initial virulence of 10^{-5}. We cultured it at 37° in defibrinated sheep blood taken under sterile conditions, making isolations in ordinary bouillon at the 24th, 48th, 72nd and 96th hour. After 24-hour culturing we diluted the bouillons with physiologic solution (each dilution made with a separate pipette) and titrated it on mice. In blood, virulence increased to 10^{-27} in 24 hours, to 10^{-25} in 48 hours and to 10^{-19} in 72 hours, and dropped below 10^{-15} in 96 hours. In contrast to the erysipelas bacteria and Listeriae, where virulence increased gradually in the event of protracted culturing, assuming at the 120th-144th hour values 900-1000 times greater than those at the 24th hour, Pasteurellae were most virulent at about the 24th hour, after which their virulence rapidly declined. By virtue of their rapid and unusually high increase in virulence the duration of this process was short.

Pasteurella pseudotuberculosis also underwent similar investigations. With initial virulence of $2 \cdot 10^{-1}$, virulence of the strain after 24-hour culturing in defibrinated (sheep and swine) blood increased 2000 fold -- to 10^{-4} -- a value close to that for the increase in virulence in 24 hours' time in a septic carcass [2].

Multiplication of Pasteurella pseudotuberculosis in blood also exhibits sizable differences in comparison with the multiplication of Past. avicida. The number of cells increased gradually -- from 50 to 500 million per ml of blood at the 12th hour, about 2.5 billion at the 24th hour, 3-4 billion at the 32nd-36th hour, whereafter it declined evenly to 500 million at the 72nd hour, i. e. it reached levels ten

times lower than the corresponding ones for Past. avicida.

The curve of postmortal multiplication of Past. pseudotuberculosis [2] is very similar to the curve of multiplication in defibrinated blood, which is an indication of a certain stability of the cells as regards the putrefactive processes. The actual formation of Bipolares septici in the case of Past. pseudotuberculosis sets in later and proceeds more slowly than in Past. avicida. Its greater lability is conspicuous in comparison with the curve of postmortal multiplication [5] and the curve of multiplication in sterile blood. Whereas in a septic carcass the greatest number of Pasteurellae is at the 12th hour after death and the disintegration of Bipolares begins at the 24th-48th hour, in sterile blood the maximum is at the 30th hour, and the minimum not before the 72nd hour.

Bacterium pyocyaneum. Under the same experimental setup with initial virulence of $3 \cdot 10^{-1}$, Bact. pyocyaneum in sheep blood increased its virulence 30 fold -- to 10^{-2} -- in 24 hours, and in swine blood 60 fold -- to $5 \cdot 10^{-3}$, with the death of the mice ensuing several days earlier (those inoculated with the culture in swine blood died on the 5th day, while those inoculated with the culture from sheep blood died, on the average, on the 10th day). This exceeds the change in virulence in the septic carcasses of guinea pigs, where the maximum value of virulence reached was $2 \cdot 10^{-3}$ [3]. This fact is probably due to the greater susceptibility of guinea pigs to this bacterium.

With 96-hour culturing of Bact. pyocyaneum in defibrinated sheep blood, virulence doubles in comparison with the the 24th hour ($5 \cdot 10^{-3}$). Thus Bact. pyocyaneum, although Gram-negative, is closer to the erysipelas type of increase in virulence.

Multiplication of Bact. pyocyaneum in defibrinated blood has the following course: about 1 billion bacteria per ml at the 5th-6th hour, about 2 billion at the 12th hour, 700-800 million at the 24th hour, whereafter it gradually increases until the 48th hour (30-40 billion) and subsequently drops to 700 million at the 72nd hour, beginning to rise again (to 2-3 billion) in subsequent hours. The curve of multiplication in blood, as compared with the Pasteurella curves, is reminiscent of the multiplication of Past. avicida in respect of the extraordinarily high number of cells and the multiplication of Past. pseudotuberculosis in respect of its maximum at barely the 48th hour. Compared with the curve

of postmortal multiplication of Bact. pyocyaneum in the blood of septic carcasses [3], it shows minimal differences.

Salmonella typhimurium was also the subject of investigation into postmortal changes of virulence, and it was of interest to see to what extent these changes would be corroborated.

With 24-hour culturing at 37° in defibrinated sheep and swine blood taken under sterile conditions, identical results were obtained: virulence increased 600 fold -- from $3 \cdot 10^{-2}$ to $5 \cdot 10^{-5}$.

Gradually the number of Salmonellae per ml of blood increased, with a maximum at the 36th hour (about 3 billion), whereafter it decreased smoothly.

Discussion of Results

The results obtained from the experiments involving the culturing of Erysipelothrix rhusiopathiae, Listeria monocytogenes, Pasteurella avicida, Pasteurella pseudotuberculosis, Bacterium pyocyaneum and Salmonella typhimurium for the purpose of tracing the rate and extent of multiplication and any changes in virulence in defibrinated blood in vitro showed that in this respect the above-mentioned pathogens behave in the same way as in the carcasses of experimental animals that died after experimental inoculations therewith. In generalized form, these results are given in Table 1.

For Past. avicida the increase is of the order of many billions of times; for Past. pseudotuberculosis in both sheep and swine blood the increase is about 2000 fold; for Bact. pyocyaneum in swine blood 60 fold and in sheep blood 30 fold; for Salm. typhimurium in both kinds of blood 600 fold; for Ery. rhusiopathiae 60 fold; for List. monocytogenes 300 fold.

As can be seen from the Table, regardless of the extraordinarily great increase in virulence the inoculated experimental animals were sick for a longer time.

In general, these data show that in blood in vitro the septicemic bacteria multiply intensely and increase their virulence to very high degrees. In view of the high multiplication of Past. pestis in the esophagi of plague-carrying fleas, which proceeds in the same way as multiplication in blood in vitro is here shown by us to proceed, it can with great probability be assumed that multiplication in the blood taken by arthropods will also entail an increase in virulence, like the

[Text continues on page 10]

[illegible]

[illegible] 24 HOURS AT 37° C

A Възбудител	B Изходна вирулентност	C Най-високата вирулентност I.	II.	D Увеличение	E Мом. ex. letalis	
Pasteurella suicida	10^{-1} глен „Топола" (Т)	свинска	10^{-11} Т	10^{-10} Т	8-и ден Т	1
			10^{-12} Ж	10^{-10} Ж	6-и ден Ж	2
	10^{-2} щам „Женол" (Ж)	овча	$10^{-[illegible]}$ Т	$10^{-[illegible]}$ Т	4-и ден Т.	3
			$10^{-[illegible]}$ Ж	$10^{-[illegible]}$ Ж	4-и ден Ж	4
Past. pseudotuberculosis	2.10^{-1}	свинска	10^{-4}	2000 пъти	7-и ден	5
		овча	10^{-4}	2000 пъти	7—10-ден	6
Bacterium pyocyaneum	$3.10^{-[illegible]}$	свинска	$5.10^{-[illegible]}$	60 пъти	5-и ден	7
		овча	$10^{-[illegible]}$	30 пъти	10-и ден	8
Salmonella typhimurium	$2.10^{-[illegible]}$	свинска	$5.10^{-[illegible]}$	600 пъти	10—11-и ден	9
		овча	$5.10^{-[illegible]}$	600 пъти	10—11-и ден	10
Er. rhusiopathiae	$2.10^{-[illegible]}$	свинска	$1,2.10^{-[illegible]}$	60 пъти	8-и ден	11
		овча	$1,2.10^{-[illegible]}$	60 пъти	8-и ден	12
Listeria monocytogenes	$3.10^{-[illegible]}$	свинска	$10^{-[illegible]}$	200 пъти	4—5-и ден	13
		овча	$10^{-[illegible]}$	200 пъти	4—5-и ден.	14

Keys to Table 1 (by columns):

A. Pathogen

B. Initial virulence

1-2. 10^{-1} "Tonev" strain (T)
3-4. 10^{-2} "Zhekov" strain (Zh)

C. Maximum virulence

I. 1-2. Swine
3-4. Sheep
5. Swine
6. Sheep
7. Swine
8. Sheep
9. Swine
10. Sheep
11. Swine
12. Sheep
13. Swine
14. Sheep

II. 2. 10^{-12} Zh
4. 10^{-12} Zh

D. Increase

2. 10^{-10} Zh
4. 10^{-10} Zh
5-14. . . . fold

E. Mom. ex. letalis

1. 8th day T
2. 6th day Zh
3. 4th day T
4. 4th day Zh
5. 7th day
6. 7th-10th day
7. 5th day
8. 10th day
9. 10th-11th day
10. 10th-11th day
11. 8th day
12. 8th day
13. 4th-5th day
14. 4th-5th day

increase in virulence which we found in blood in vitro. Special investigations are in prospect to study this problem. Bloodsucking arthropods will be released on inoculated experimental animals during the stage of bacteriemia. The rate and extent of multiplication will be traced in blood from their esophagi, while virulence will be traced in cultures isolated from them at fixed intervals of time by titration on experimental animals.

Conclusions

On the basis of the experiments here conducted the following conclusions can be drawn:

1. The investigated strains of Erysipelothrix rhusiopathiae, Listeria monocytogenes, Pasteurella avicida, Pasteurella pseudotuberculosis, Bacterium pyocyaneum and Salmonella typhimurium in 24 hours' time increase their virulence in defibrinated sheep and swine blood up to values close to or identical with those which virulence reaches in septic carcasses.

2. The same strains if cultivated for a longer time in defibrinated sheep and swine blood increase their virulence several times more than in carcasses. This is an indication of the inhibitory effect which the putrefactive processes have on virulence and which begins to be manifested in the later stage of these processes.

Past. avicida constitutes an exception. Its maximum virulence occurs by the 24th hour, followed by a rapid decline -- one more indication of its extraordinary lability.

3. Differences between the increase of virulence if cultured in sheep blood and if cultured in swine blood are revealed only in the case of Bact. pyocyaneum. Strains of this bacterial species cultivated in swine blood doubled their virulence as compared with strains cultivated in sheep blood and isolated from carcasses, and inoculated mice died twice as rapidly.

4. White mice inoculated with septicemic pathogens whose virulence had intensified following cultivation in defibrinated blood died later in almost all cases than those inoculated with cultures isolated post mortem from septic carcasses.

5. Multiplication of the investigated bacteria in defibrinated sheep blood showed values closer (Ery. rhusiopathiae, List. monocytogenes, Past. pseudotuberculosis) to

or more diverse (Past. avicida) than those in septic carcasses. There is a general tendency on the part of the Bipolares septici to balloon and be lysed in the defibrinated blood more slowly. This is emphasized especially well in the case of Past. avicida and is a sign of its high susceptibility to putrefactive processes and their products.

6. The results obtained from the investigations into the multiplication and increase in virulence in blood in vitro of six species of bacteria from the group of septicemic and blood pathogens gives us the basis for proceeding to the next stage of work from this point of view, viz. to undertake investigations to trace the manner in which septicemic and blood pathogens multiply and perhaps increase in virulence in infinitesimal quantities of blood taken by bloodsucking arthropods in transmissive infections.

Study of this question is of extraordinarily great value in order to clarify the actual role and significance of Arthropoda as a factor not only in disseminating infectious diseases of this group but also in increasing the amount and intensifying the virulence of the infectious principle.

Submitted to press 26 November 1964.

BIBLIOGRAPHY

1. Merchant, I. A., and R. A. Packer, Veterinary Bacteriology and Virology, Ames, Iowa, USA, 404, 1956.

2. Toshkov, Al. and D. Velyanov, "Multiplication and Virulence of Septicemic and Blood Pathogens in the Organism of Infected Animals and their Carcasses. Report II, Izvestiya na Mikrobiol. inst. na BAN (News of the Microbiological Institute of the Bulgarian Academy of Sciences), 16, 1964.

3. Toshkov, Al. and D. Velyanov, Izvestiya na Mikrobiol. inst. na BAN (News of the Microbiological Institute of the Bulgarian Academy of Sciences), 16, 1964.

4. Toshkov, Al. and D. Kaloyanov, Izvestiya na Mikrobiol. inst. na BAN (News of the Microbiological Institute of the Bulgarian Academy of Sciences), 16, 1964.

5. Toshkov, Al., As. Toshkov and G. Kamburov, Izvestiya na Mikrobiol. inst. na BAN (News of the Microbiological Institute of the Bulgarian Academy of Sciences), 16, 1964.

SUMMARY

The authors traced the multiplication and change in virulence of Erysipelothrix rhusiopathiae, Listeria monocytogenes, Pasteurella avicida, Pasteurella pseudotuberculosis, Bacterium pyocyaneum and Salmonella typhimurium when these were cultured in defibrinated sheep and swine blood taken under sterile conditions.

Multiplication in defibrinated blood shows values close to the values in septic carcasses. The maximum number of Ery. rhusiopathiae and List. monocytogenes bacteria per milliliter of blood occurs in 24 hours (about 2.5 billion); for Past. avicida the maximum is established by the 30th hour and the number of microorganisms per ml reaches huge quantities -- 30-40 billion. The situation is approximately the same with Bact. pyocyaneum as well, except for a maximum at the 48th hour. Past. pseudotuberculosis and Salmonella typhimurium have an intermediate ranking in this respect.

The authors also established differences between the rate of ballooning and disintegration of Bipolares in blood and in septic carcasses, most markedly pronounced in the case of Past. avicida. The more rapid formation and lysis of Bipolares in carcasses are due to the great susceptibility of Pasteurellae to putrefactive processes and their products.

The increase in virulence, determined by the difference between initial virulence and virulence after culturing in blood in vitro for 24 hours at 37° C, is specific for each of the six investigated species of bacteria. For Past. avicida virulence increases many billion fold; for Past. pseudotuberculosis about 2000 fold; for Bact. pyocyaneum -- in blood -- 60 fold and in sheep blood 30 fold; for Salm. typhimurium 600 fold; for Ery. rhusiopathiae 60 fold and for Lis. monocytogenes about 300 fold.

Regardless of the exceptionally great increase in virulence the experimental animals in almost all cases die later than animals inoculated with cultures isolated post mortem from septic carcasses.

These same strains when cultured for an even longer time in defibrinated sheep and swine blood continue to increase in virulence up to values several times greater than those in carcasses. This is an indication of the inhibitory effect which advanced putrefactive processes in carcasses, together with their products, have on virulence.

All these results provide a basis for undertaking investigations in order to trace the extent to which the numbers and virulence of septicemic pathogens increase in blood -- even in the infinitesimal quantities of blood taken by blood-sucking arthropods in transmissive bacterial infections. This would clarify experimentally the actual part played by, and the significance of, Arthropoda in the epizootiology, epidemiology and natural foci of a considerable number of infectious diseases.